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Plazomicin is effective in a non-human primate pneumonic plague model

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ABSTRACT

The efficacy of plazomicin for pneumonic plague was evaluated in a non-human primate model. African Green monkeys challenged with a lethal aerosol of *Yersinia pestis* [median (range) of 98 (15–331) LD_{50s}] received placebo ($n = 12$) or 'humanized' dose regimens (6.25, 12.5 or 25 mg/kg every 24 h) of plazomicin ($n = 52$) after the onset of fever for a duration of 5 or 10 days. All animals treated with placebo died, while 36 plazomicin-treated animals survived through study end. The majority (33/36) were either in the 10-day (high-/mid-/low-dose) or 5-day high-dose groups. The findings suggest an exposure range of plazomicin for treatment of pneumonic/bacteremic *Y. pestis* infection in humans.

1. Introduction

Plazomicin (plazomicin sulfate) is a novel compound in the aminoglycoside class of antibiotics that is being developed for the treatment of serious Gram-negative infections. Plazomicin contains structural modifications that allow it to maintain activity in the presence of the common aminoglycoside-modifying enzymes (AMEs) that inactivate currently marketed aminoglycosides[1] (Fig. 1). Furthermore, plazomicin has demonstrated in vitro activity against clinical isolates that possess a broad range of resistance mechanisms, including beta-lactamases and fluoroquinolone target site mutations that limit the utility of other classes of antibiotics. At the time of this publication, plazomicin is being studied in two phase 3 clinical trials (NCT01970371 and NCT02486627), one of which is specifically focused on serious infections due to carbapenem-resistant Enterobacteriaceae (CRE), a family of multidrug resistant (MDR) pathogens that has been identified as an urgent health need.[2]

In partnership with the Biomedical Advanced Research and Development Authority (BARDA), plazomicin is also being investigated for the treatment of two diseases caused by potential bioterrorism agents, pneumonic Tularemia, caused by *Franciscella tularensis*, and pneumonic plague, caused by *Yersinia pestis*. In this article, we describe the evaluation of plazomicin in a primate model of pneumonic plague.

Y. pestis is considered to be one of the most threatening bioweapons due to the virulence of the bacterium, the availability of virulent strains in natural environmental reservoirs, the ease of preparation of aerosols and the rapid onset of symptoms and death associated with primary pneumonic plague.[3] Also, unlike diseases such as anthrax, pneumonic plague has a greater potential to spread from person-to-person.[3,4] While a handful of plague cases are reported annually in the United States, they are predominantly the bubonic form, which is generally caused by contact with an infected animal.[5] The severity of disease and high associated mortality of primary pneumonic plague is not adequately captured in these bubonic plague patients. The sparse case reports of primary pneumonic plague that are available in the literature indicate that it is a rapidly progressing and most often lethal disease, even with antibiotic treatment.[3] While it is fortunate that the incidence of pneumonic plague is low, there is a critical need for new therapies in the event of an attack with weaponized, aerosolized plague.

Levofloxacin and moxifloxacin are the only two FDA-approved antibiotics for the treatment of primary pneumonic plague. Both were recently approved (2012 and 2015 respectively) using an animal model similar to the one described in this study.[6,7] While

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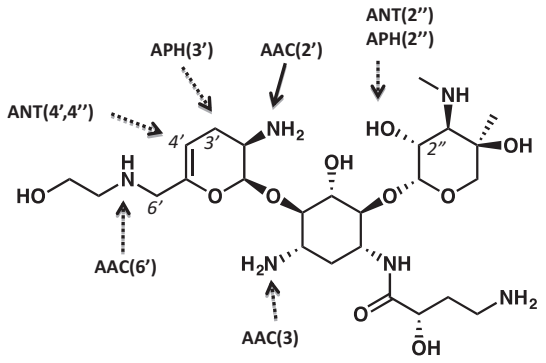

Figure 1. Plazomicin structure and AME families from Gram-negative and Gram-positive organisms. AME families shown with dotted arrows cannot modify plazomicin. AAC, aminoglycoside acetyltransferase; ANT, aminoglycoside nucleotidyltransferase; APH, aminoglycoside phosphotransferase.

it is encouraging that two agents are now approved for treatment, both are members of the fluoroquinolone class of antibiotics and therefore share common resistance mechanisms. In the event that a fluoroquinolone-resistant strain was disseminated, either naturally or intentionally, the efficacy of both approved drugs would be compromised. The ideal armamentarium for any infectious agent would consist of several drugs possessing different modes of action. Plazomicin is an aminoglycoside antibiotic that targets the bacterial ribosome and therefore has a different cellular target and mechanism of bacterial killing than the fluoroquinolones. Plazomicin has also been chemically modified to maintain activity in the presence of many of the AMEs that inactivate older aminoglycosides (Fig. 1).[1] Based on its differential mode of action compared with the fluoroquinolones and its ability to kill strains resistant to other members of the aminoglycoside class, plazomicin would significantly add to our armamentarium against *Y. pestis*.

The United States Food and Drug Administration's (FDA) 'animal rule' enables a path to regulatory approval for new therapies directed at rare infectious agents by establishing efficacy in animals when the study drug cannot be feasibly/ethically tested in humans.[8] Therefore this study aimed to test the hypothesis that plazomicin could improve the survival of non-human primates after a lethal aerosol dose of *Y. pestis* strain Colorado 92.[9] The African Green monkey (AGM) was chosen as the test system predominately due to historical precedent. AGMs are highly susceptible to *Y. pestis* aerosol infection and the timing and evolution of disseminated disease following aerosol exposure is similar to humans.[10] AGM was the primate species utilized in the levofloxacin and moxifloxacin studies to support approval,[6,7] and the FDA advisory committee from the levofloxacin approval unanimously agreed the model is able to 'provide substantial evidence of efficacy. . .for the treatment of humans with pneumonic plague'.[11,12]

In addition, an important feature of this study is that treatment with plazomicin was delayed until the disease advanced from a primary pneumonia to a systemic disease hallmarked by spread of *Y. pestis* from the lungs into the bloodstream and surrounding organs, a process that takes between 2 and 3 days. In the event of an outbreak or intentional release of aerosolized plague, it is likely that many exposed individuals will not present to a medical facility for treatment until they exhibit clear signs and symptoms of infection. We therefore sought to evaluate the efficacy of plazomicin under these more challenging conditions in which the disease has already progressed.

The current phase 3 plazomicin studies are using a once-daily 15 mg/kg dose, administered as a 30-min intravenous (IV) infusion, with dose adjustments required for patients with compromised renal function.[13] Based on a population PK model generated from healthy volunteer data and data collected in patients with complicated urinary tract infections or acute pyelonephritis, the mean daily steady state area under the curve (AUC_{24}) associated with this dose is 262 mg/L × h.[14] We sought to determine if this AUC_{24} was sufficient to treat pneumonic plague in AGMs.

We also investigated the impact of duration of plazomicin therapy on outcome. There are very few antibiotic studies that examine the optimal duration of therapy and, as a result, one of the biggest knowledge gaps in the antibacterial field is a clear understanding of the importance of treatment duration on outcome.[15–17] The AGM study provides an opportunity to empirically examine the impact of treatment duration on outcomes in otherwise healthy animals with a life-threatening, multi-organ infection with a large bacterial burden when therapy is initiated, akin to the disease state in critically ill patients with pneumonia and associated bacteremia/sepsis.

2. Material and methods

2.1. Overview of plague study design

Plazomicin was evaluated in three separate studies referred to below as FY-105A, FY-105B and FY-036A. These studies were similar in design but each explored different aspects of plazomicin dosing and duration of therapy. Due to animal handling limitations, each study was separated into two exposure cohorts. In total, 64 animals were evaluated across the six cohorts. A placebo group was included in each cohort and all attempts were made to ensure that each cohort was handled identically. Briefly, AGMs were anesthetized and exposed, head only, via aerosol to lethal doses of *Y. pestis*. Treatment was delayed until the disease had progressed from an isolated pneumonia to a multi-organ, systemic disease marked by spreading of the bacteria through the bloodstream to other body sites and the occurrence of sustained fever. Since our intent was to treat animals when they were bacteremic, and knowing that the presence of bacteremia takes >24 h to confirm, we required a surrogate marker. It had previously been established that the systemic phase of disease is associated with fever.[10] Therefore, animals were continuously monitored for development of fever via implanted telemetry. Treatment with placebo or 'humanized' doses of plazomicin (6.25, 12.5 or 25 mg/kg 30-min infusion every 24 h) was initiated within 6 h of onset of sustained fever and was continued for 5 or 10 days, depending on the study cohort. The total observation period post-aerosol challenge was between 28 and 30 days (Fig. 2). Blood samples were taken to evaluate plazomicin PK on the first, third and last day of treatment and compared with results from healthy animals. In addition, blood was sampled pre-challenge and at time of death or study end, to evaluate serum chemistry, hematology and quantitative bacteriology. Upon death, euthanasia due to meeting moribund criteria, or euthanasia due to study end, histopathological examination of multiple organs and determination of bacterial burden were performed.

2.2. Animal assurance and procedures

All studies complied with all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR Parts 1, 2 and 3) and the Guide for the Care and Use of Laboratory Animals (8th Ed., 2010, National Academies Press, Washington, DC). All in-life portions of the studies were performed in Lovelace Respiratory Research Institutes (LRRI) facilities, which are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

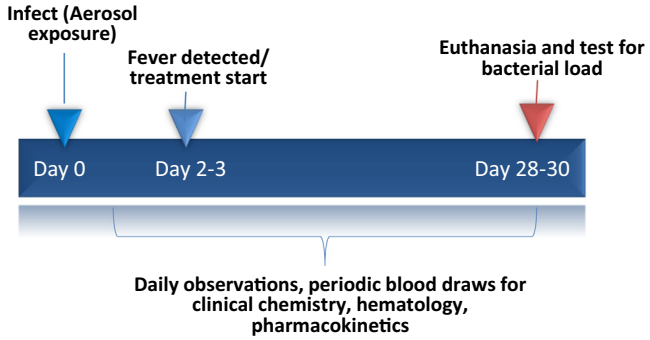

Figure 2. Study timeline overview.

AGMs were jacketed and conditioned to a restraint collar, poles, and chair and limb restraints. Animals were quarantined for at least 30 days prior to study acceptance, and only animals of acceptable health were included. Prior to challenge group allocation, each animal underwent a complete physical examination by a staff veterinarian or veterinary technician under the supervision of a veterinarian. The examination included complete blood count, serum chemistry and fecal ova and parasite determination. Additionally, AGMs were screened prior to arrival at LRRI for tuberculosis, measles, simian immunodeficiency virus, simian T-cell leukemia virus, simian retro virus (SRV1, 2, 3, 4, D) and simian agent 8.

T34 telemeters (Konigsberg Instruments, Inc., Pasadena, CA) were secured to the abdominal wall within the peritoneum for continuous monitoring of body temperature, respiratory rate and heart rate. All animals also had a venous access catheter aseptically implanted into the right femoral vein and tunneled through the right flank and back, emerging through the skin of the upper mid-back. Catheters were maintained per LLRI's standard procedures for indwelling vascular catheter care and maintenance in nonhuman primates. Animals were moved into the ABSL-3 facility at least 5 days prior to challenge with *Y. pestis*.

2.3. Bacterial challenge

Y. pestis Colorado 92 was obtained from the Centers for Disease Control and Prevention (CDC, Fort Collins, CO) via C. Rick Lyons (School of Medicine, University of New Mexico, Albuquerque, NM) and characterized in the LRRI Animal Biosafety Level (ABSL)-3 microbiology laboratory. Seed and working bacterial stocks were stored frozen (−70 to −90 °C). *Y. pestis* Colorado 92 nebulizer suspensions were prepared fresh on each exposure day. A single working stock vial was removed from frozen storage and used to inoculate tryptose blood agar base (TBAB) slants. The TBAB slants were incubated at 28 °C ± 2 °C for 72 h. Phenotype was verified on Congo Red agar. After incubation, cells from the TBAB slants were suspended into 2 mL of 1% peptone per slant. The bacterial suspensions were collected into a 50 mL conical tube and then centrifuged at 4100 rpm ± 200 rpm, 4 °C ± 2 °C for 20 min. The *Y. pestis* pellet was suspended in 4 mL of 1% peptone and mixed using a vortex mixer. The optical density at 600 nm (OD600) was determined and the culture adjusted to the target density.

Animals were fasted overnight and anesthetized by intramuscular injection of 2–6 mg/kg Telazol. The exposure system was a Class 3 biosafety glovebox with a head-only exposure unit and plethysmography measuring respiratory frequency, tidal volume and minute volume during the exposure. The bacterial suspension was nebulized using a Collison MRE-3 (MRE-3 jet, BGI, Inc., Waltham, MA) nebulizer and delivered to the anesthetized animal which was breathing freely. The concentration of the bacteria in the nebulizer suspension was estimated (by OD600) and set to deliver a target concentration of bacteria per minute with the goal of achieving an infectious load in the lungs of approximately 35,000 ± 17,500 colony forming units (CFUs) of *Y. pestis* which equates to 100 ± 50 times the median lethal dose (LD_{50}).[18]

The bacteria-containing aerosol was sampled directly into an all glass impinger (AGI; Ace Glass, Inc., Vineland, NJ) drawn from the head only exposure apparatus, downstream from the primate's nares. Generator and impinger suspensions (pre- and post-bioaerosol) were serially diluted and plated on tryptic soy agar to determine bacterial titers (CFU/mL) and quadrant-streaked on Congo Red agar to confirm culture pathogenicity by colony morphology. Both samples were incubated at 28 °C for 72 h prior to analysis. The target particle size was 1–3 μm; particle size was determined using a GRIMM Portable Aerosol Spectrometer Model 1.109 (GRIMM Aerosol Technik GmbH & Co. KG, Ainring, Germany). The duration of the exposure was calculated based on the rate of bacteria delivery and the total volume of air inhaled by the animal (target range was 3.5 ± 0.5 L). Inhaled aerosol dose (CFU inhaled) was calculated after direct measurement of inhaled volume (from plethysmography) and bacterial aerosol concentration was determined from the impinger sample. The calculation used the formula: Dose = (C × V), where C is the concentration of viable pathogen in the exposure atmosphere, and V is the volume inhaled.

2.4. Plazomicin pharmacokinetics (PK) study in healthy animals and dose justification for efficacy study

The intent of the efficacy studies was to determine if the current clinical plazomicin dose is effective in the AGM model. Because of differences in clearing organ function per unit body weight across species,[19] higher doses are generally required for smaller animals to generate the same drug exposure. To address this, a repeat-dose PK study was conducted in compliance with the FDA 21 CFR Part 58 Good Laboratory Practice (GLP).[20] Eight healthy AGMs underwent a complete physical exam and were randomized (stratified by body weight) to two dose groups receiving total daily plazomicin doses of 25 or 35 mg/kg/day. Animals were administered plazomicin by IV infusion twice daily (target interval between AM and PM infusions was 12 h) for 7 consecutive days. Blood was collected at multiple time points during each of the 7 days of dosing and analyzed for plazomicin concentration by LC–MS/MS. PK analysis was performed using WinNonLin Professional, Version 5.0.1, model 202. Additional blood was collected each morning prior to dose administration for creatinine values on days 1–7 and analyzed using the Hitachi Modular Analytics Clinical Chemistry System (Roche Diagnostics, Indianapolis, IN).

2.5. Drug administration

Sixty-four AGMs were randomized into six exposure cohorts using Microsoft Excel's® (Microsoft, Redmond, WA) random number generator. Exposure order was randomly assigned per gender using Provantis® (Instem LSS Ltd, Staffordshire, England) or Microsoft Excel's® random number generator, alternating between the sexes. Animals were assigned to treatment arms by exposure order.

Treatment with plazomicin or placebo (saline for injection, the diluent for plazomicin) was initiated within 6 h of the first recorded sustained fever. For studies FY-105A and FY-105B, this was defined as a temperature of >39 °C sustained for 1 h. Based on the observation of variable baseline temperatures across animals in the first two studies, the fever definition was changed in study FY-036A to an increase of 1.5 °C over baseline sustained for 1 h. The baseline temperature was determined by averaging each individual animal's temperature in hourly intervals (to account

for the diurnal cycle) for a minimum of 3 days prior to aerosol challenge.

Treatments were administered through the surgically implanted catheter in the femoral vein via a syringe pump in a total volume of 1 mL/kg (based on day −1 body weight), over 30 ± 5 min every 12 ± 1 h. To mimic the human PK of plazomicin in the AGMs (e.g., 'humanized' once daily dosing), alternating dose solutions that contained 90% and then 10% of the total daily dose (6.25, 12.5 or 25 mg/kg), were administered.[21,22] This was done to correct for the more rapid clearance in AGMs compared to humans and to more closely approximate the shape of the PK curve and trough drug levels in human patients given a once-daily infusion. A similar strategy was followed in the pivotal levofloxacin plague studies.[22] For each animal, the first two daily primary (90%) dosages (dose 1 and 3) were administered 24–25 h apart. A longer time interval (24–28 h) was allowed between dose 3 and 5 to enable synchronization of animal treatments.

2.6. Clinical observations

Pre-exposure clinical observations were performed with special attention to normal behavior, presentation, and food intake to provide baselines for each individual animal post-exposure. For up to 30 days following challenge, Provantis-defined clinical observations were performed by LRRI according to standard operating procedures,[23] noting respiratory distress, neurological symptoms, provoked and unprovoked behavior, food intake and body weight trends, appearance/posture and gastrointestinal/urogenital abnormalities.

Between 2 and 6 days post-exposure, animals had an increased likelihood of reaching a moribund state. Therefore, during that period, animals were monitored via telemetry for changes in body temperature, respiratory rate and heart rate at least every 4–6 h. During the treatment phase, observations were timed to occur during the test article infusion periods. For the remainder of the study, clinical observations were performed twice daily.

2.7. Telemetry observations

Body temperature (abdominal core temperature; °C), respiratory rate (thoracic cavity movement; breaths/min), and heart rate (derived from the R–R interval via the electrocardiogram leads in a Lead II configuration; beats per min) were continuously recorded by implanted Konigsberg T34 telemetry devices (Konigsberg Instruments, Inc., Pasadena, CA). Telemetry data were averaged (derived) into 5-min values for real time data evaluation during the in-life phase of the study. During the clinically critical phase of the study (days 2–6), where the onset of infection (febrile status) and likelihood of reaching euthanasia criteria is highest, telemetry data were assessed every 4–6 h. In one instance, a telemetry failure occurred. For this animal, a rectal temperature was used to determine febrile state.

2.8. Clinical chemistry, hematology, bacteremia determination and pharmacokinetics

Blood was drawn from animals for clinical chemistry, hematology and quantitative bacteremia determination pre-challenge, pre-first treatment with placebo or plazomicin, prior to fifth and ninth or nineteenth treatment and prior to euthanasia. For clinical chemistry, blood was collected into a serum separator tube, allowed to clot and then centrifuged for at least 10 min at 2500–3000×*g* at ambient temperature. Serum parameters were determined using a cobas c311 chemistry analyzer (Roche Diagnostics, Indianapolis, IN). For hematology analysis, blood was collected into a tube containing ethylenediaminetetraacetic acid (EDTA) and a glass slide smear was made. Hematology parameters were determined using an Advia®120 (Bayer Corporation, Diagnostic Division, Tarrytown, NY).

To determine bacterial load, blood (target volume 1.2–1.5 mL) was collected into a tube containing EDTA, and serial dilutions of the sample were plated. The remaining undiluted sample (up to 1 mL) was plated on multiple plates (maximum volume of 200 μL per 100 mm plate) to achieve a detection limit of approximately 1 CFU/mL.

To determine plazomicin levels, blood (target volume ⩽ 1 mL) was collected into K2EDTA tubes and centrifuged (2500–3000×*g* at ambient temperature) to separate plasma. The plasma was centrifuge-filtered through a 0.2 μm Nanosep MF centrifugal filter (Pall Corp., East Hills, NY) at 13,000×*g* for ⩾20 min to sterilize the plasma samples for subsequent removal from the ABSL3 facility for analysis. A 10% aliquot of each sample was cultured to confirm sterility. The remainder of the sample was stored frozen at −70 ± 10 °C until sterility testing was completed and then shipped to Alturas Analytics (Moscow, ID) for HPLC/MS/MS analysis. Pharmacokinetic samples were drawn from placebo control animals on day 1 only at ⩽10 min after the end of the primary infusion (peak level). Plazomicin-treated animals were tested pre-dose (collected ⩽30 min prior to the primary infusion; trough) and ⩽10 min after the end of the primary infusion (peak level) on treatment days 1, 2, 3, 5 and additionally, for long-duration treatment groups, day 7 and 10.

2.9. Pathology and cause of death

Moribund or study end (28–30 days post-challenge) animals were anesthetized with an intramuscular injection of ⩾10 mg/kg ketamine and euthanized by IV administration of 87 mg/kg pentobarbital and 11 mg/kg phenytoin. Observation thresholds for euthanasia included temperature >1.5 °C above baseline ('fever') followed by a consistent decline to >1.5 °C below baseline, heart rate >200 beats/min or higher for >24 h, which may or may not be followed by a rapid decline, deep labored breaths with obvious excessive work of breathing; >60 respirations/min for >24 h with this breathing pattern, and physical observations (seizures [prolonged (>10 min) or recurrent (>3 instances of <10 min], or too weak to climb onto perch or falling off perch, or constant hunched posture and unresponsive to stimulation and refusal to eat any offered food >48 h). The cause of death for each animal was established by a board certified veterinary pathologist. Samples of selected tissues (lung, liver, spleen, tracheobronchial lymph nodes, kidney, brain, heart and any observed gross lesions) were taken at necropsy, weighed and analyzed for the presence of *Y. pestis* by quantitative culture. Tissue sections were fixed in 10% neutral-buffered formalin, embedded in paraffin, processed by routine histological methods and stained with hematoxylin. Slides were evaluated by a qualified veterinary pathologist.

In study FY-036, *Y. pestis* was collected from the tissues of animals that did not survive until study end and suspended in cryomedium (brain heart infusion broth + 20% glycerol) and stored at −80 ± 10 °C for plazomicin MIC determination. No bacteria were detected at study termination (day 28–30 post-challenge) in any animal across the three studies.

3. Results

3.1. Plazomicin Pharmacokinetics (PK) in healthy animals

Plazomicin PK parameters are summarized in Table 1, and were similar on day 1 and 7. One female AGM in the 25 mg/kg cohort had a significantly lower AUC_{24} on day 1 (116 mg/L × h) compared

Table 1
Pharmacokinetics of plazomicin and serum creatinine levels in 8 healthy AGMs – day 1 versus day 7

Dose group	Day	C_{max} (mg/L)	AUC_{24} (mg/L * h)	$T_{1/2}$ (h)	CL (L/h/kg)	Serum creatinine (mg/dL)	V_{ss} (L/kg)	Human equivalent dose[a] (mg/kg)
25 mg/kg	1	96 ± 32	185 ± 65	1.8 ± 0.1	0.13 ± 0.05	0.55 ± 0.14	0.33 ± 0.14	12
	7	106 ± 15	208 ± 45	1.8 ± 0.1	0.11 ± 0.02	0.55 ± 0.13	0.36 ± 0.08	
35 mg/kg	1	176 ± 24	306 ± 35	1.7 ± 0.1	0.10 ± 0.01	0.60 ± 0.05	0.26 ± 0.04	18
	7	185 ± 13	319 ± 45	1.7 ± 0.1	0.10 ± 0.01	0.60 ± 0.13	0.31 ± 0.03	

All values shown are median ± the standard deviation.
[a] Based on AUC_{24} and C_{max}, the estimated dose equivalent to generate the same exposure in humans AUC_{24}, area under the concentration time curve from time zero to 24 h; CL, systemic clearance; C_{max}, maximum plasma drug concentration; $T_{1/2}$, elimination half-life; V_{ss}, steady-state volume of distribution.

to the other animals, reducing the mean median AUC_{24} from 215 to 185 mg/L × h. The day 7 AUC_{24} for this animal was also low but was more in line with values from other animals, reducing the median AUC_{24} from 220 to 208 mg/L × h. There is no obvious physiological explanation for the lower exposure in this AGM based on review of available health history and other covariates, although she had the lowest bodyweight at study start. Serum creatinine levels for each individual animal were either unchanged or dropped by 0.1 mg/dL from day 1 to day 7.

The PK data indicate that to generate a median AUC_{24} similar to 15 mg/kg plazomicin in humans, the dose is approximately 31 mg/kg/day in AGMs. We proceeded to efficacy studies with 25 mg/kg as the top dose with the intention of slightly under-dosing (by approximately 20%) in this model. By doing this, we increased the probability of a high (⩾90%) pharmacokinetic/pharmacodynamic (PK/PD) target attainment in future analyses when PK variability in critically ill patients[24–26] will be considered.

3.2. Comparison of plazomicin PK in healthy versus infected animals

In general, plazomicin PK was similar in *Y. pestis*-infected AGMs compared to healthy animals. A representative dataset of the six animals from study FY-105A administered the 25 mg/kg daily dose compared to the results from healthy animals is shown in Figure 3.

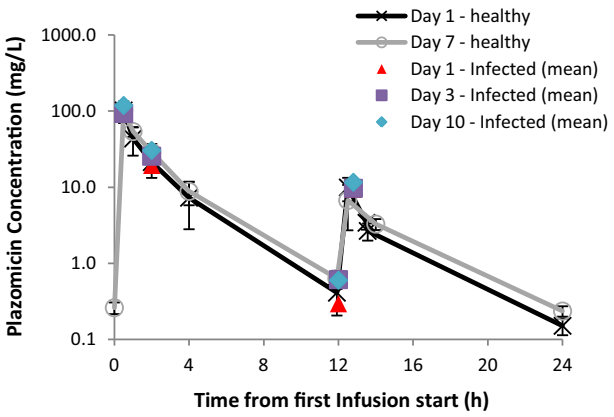

Figure 3. Healthy versus *Y. pestis*-infected AGM PK. Mean ± standard deviation from four healthy AGMs administered 25 mg/kg plazomicin for 7 days (day 1 vs. day 7 plotted as separate series). PK time points from day 1, 3 and 10 of dosing from six *Y. pestis*-infected AGMs administered 25 mg/kg plazomicin for 10 days.

3.3. Time to fever/death does not correlate with increased aerosol exposure in the range tested

Across the 64 animals, the inhaled bacteria exposures ranged from 15 to 331 times LD_{50} (target was 100 ± 50 LD_{50}). Fourteen animals fell outside the upper target range and six animals fell outside the lower target range. Study FY-105a in general had higher inhaled exposures but disease progression was similar to the other studies. There was no correlation between inhaled exposure and time to fever within the range tested (Fig. 4a). This is not particularly surprising given the rapid (~1.5 h) division time of *Y. pestis* in vivo[27] relative to the time to fever (48–72 h); small differences in starting bacterial population are quickly erased. All six animals that received challenge burdens lower than the intended target (15–48 times the LD_{50}) recorded a fever within the range of the other animals (60.2–74.9 h) and all six were confirmed positive for bacteremia before they were placed on therapy.

Similar to time to fever, there was no correlation between aerosol exposure and time to death in placebo-treated animals (Fig. 4b). The time from fever to death was more consistent than the association of either time to fever or time to death with inhaled bacterial exposure (Fig. 4c), indicating that while the level of initial bacterial exposure does not necessarily correlate with disease progression in the early stages of infection, once fever is established, time to death from onset of fever is more consistent (44.5 ± 12 h). Therefore, within the aerosol exposure range tested (15–331 LD_{50}) disease progression is highly consistent.

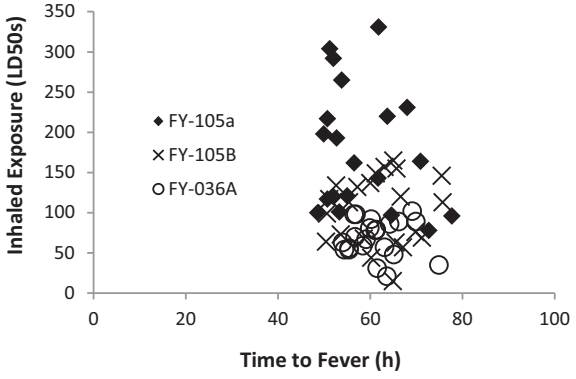

Figure 4a. Time to fever versus aerosol exposure in placebo- and plazomicin-treated animals. Series represent the three separate studies.

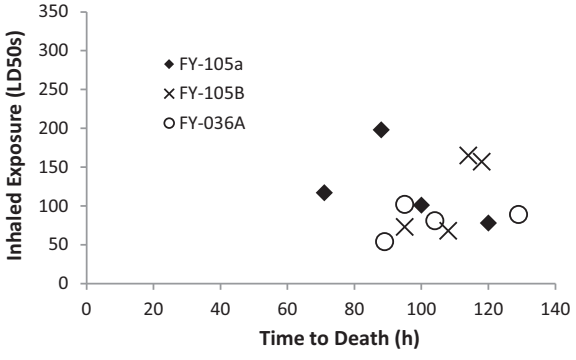

Figure 4b. Time to death versus aerosol exposure in placebo-treated animals. Series represent the three separate studies.

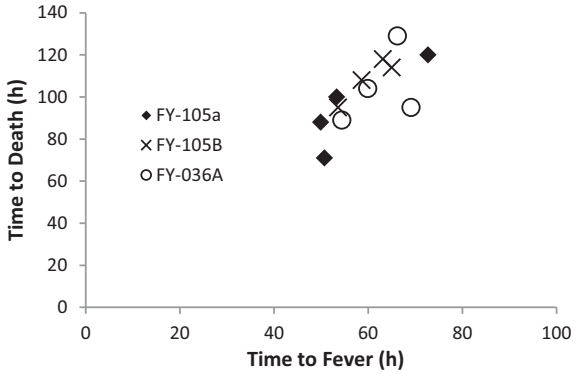

Figure 4c. Time to fever versus time to death in placebo-treated animals. Series represent the three separate studies.

A goal of the study was to initiate treatment when animals were bacteremic, using fever as a surrogate marker. Of the 64 animals across all studies, 60 were confirmed to have been bacteremic prior to treatment, supporting the use of fever as a good indicator of bacteremia in this model. One sample was not evaluable (animal B6436), although this animal eventually succumbed to the disease, indicating that it was infected. Animals 7972, 7643 and 7908 had no detectable bacteria in their blood prior to treatment initiation, were treated with plazomicin, and survived to study end. Two of these animals were in the 25 mg/kg, 10-day duration treatment group and the third was in the 12.5 mg/kg, 10-day duration treatment group. All three animals received an LD_{50} exposure within the target range. At 28-day necropsy, all three animals had histopathological evidence of a prior infection with resolving interstitial and bronchiolar mononuclear cell infiltrations in all lung lobes and additional evidence of substantial local challenge infection that had resolved (see below). It remains unclear whether these three animals gave false-negative blood cultures or whether they were truly not bacteremic at the time of treatment initiation.

3.4. Disease progression of placebo-treated animals

In total, across the six aerosol exposure cohorts, there were 12 placebo-treated animals. *Y. pestis* challenge doses ranged from 54–198 LD_{50} in these animals and the median time to fever was 59.3 h. Placebo infusions were initiated 0.5–5.9 h post establishment of fever. All blood samples drawn prior to the first placebo infusion were positive for bacteremia with a median bacterial density of 1.5×10^4 CFU/mL. All placebo-treated animals succumbed to the disease within 71–129 h post-aerosol challenge. The range of survival times between placebo infusion start and death was 17–61 h, therefore some animals started therapy <24 h before death, illustrating an advanced state of decline when therapy was initiated.

Five animals met moribund criteria (71–120 h post-aerosol exposure) and were euthanized while the other seven died (88–129 h post-aerosol exposure) prior to meeting the pre-defined euthanasia criteria. At time of death, extremely high densities of *Y. pestis* were observed in all tissues tested (Table 2). The median bacterial burdens in lung, spleen, liver, kidney and tracheobronchial lymph nodes were 7.7×10^8, 1.6×10^9, 5.1×10^8, 7.1×10^7 and 7.7×10^9 CFU/g of tissue, respectively. Due to gross pathological findings early in the first study, the protocol was amended to include analysis of heart and brain tissues, and median bacterial burdens in these tissues were 2.6×10^7 CFU/g and 1.9×10^6 CFU/g, respectively.

Comparison of a selected subset of clinical chemistry and hematology parameters at baseline and time of death in those animals in whom samples were available (Table 3) revealed multiple organ failure with declining kidney function (increases in blood urea nitrogen and serum creatinine) and liver function (increases in alanine aminotransferase and gamma glutamyl-transferase). Significant increases in serum lactate dehydrogenase and white blood cell count indicate extensive tissue damage and inflammation.

Based on pathological findings, the assigned cause of death for 11 of the 12 placebo-treated animals was sepsis with or without direct contribution of concurrent pneumonia. The cause of death in the twelfth animal, B6394, was sepsis exacerbated by aspiration pneumonia, which was attributed to overall worsening condition and sepsis.

3.5. Overall survival of plazomicin-treated animals

Relative to placebo-treated animals, all animals receiving plazomicin, regardless of dose or duration of therapy, showed significantly increased survival time (Figs. 5a5b and Table 4). Among the plazomicin-treated groups, survival to study end improved with both increased dose of plazomicin as well as increased duration of therapy. Shorter duration therapy with 25 mg/kg plazomicin improved survival to study end to 69%. The short duration 12.5 mg/kg and 6.25 mg/kg doses only protected 33% and 17% of animals, respectively, and were the only dose/duration regimens that did not provide a statistically significant improvement in survival to study end relative to placebo control, although they did significantly extend survival time. Across all studies, three animals died very early while still on therapy (described in detail below); therefore they are not valid data points to include in a comparison of therapy durations since they did not complete treatment. Removing those animals revealed that for the high-dose group, results were similar for 5- versus 10-day treatment but still trended toward inferiority for the mid- and low-dose group (Table 5). The highest survival rates were observed with 10 days of dosing, with even the lowest dose of plazomicin providing significant benefit.

3.6. Disease progression of plazomicin-treated animals

3.6.1. Deaths on therapy

Three animals died while receiving plazomicin therapy. In the 25 mg/kg 5-day treatment group, animals B6469 and B6436 succumbed 57 and 14 h after initiating therapy, respectively. One 12.5 mg/kg 5-day treatment animal, B6873, also succumbed after receiving all but one of its infusions.

Animal B6436, which only received a single humanized dose of plazomicin, had moderate to severe, locally extensive, sublobar and lobar acute inflammation in multiple lung lobes and mild, locally extensive acute inflammation in the right middle lobe. There was pronounced hemorrhage with alveolar edema and/or fibrin exudate in some areas and associated destruction of the alveolar walls. Viable bacteria were not detected by histologic examination in areas of acute inflammation or in blood vessels or lymphatics. High power magnification and image enlargement detected pale staining bacteria-like forms that may have been non-viable bacteria. Low bacterial numbers ($\sim 10^3$ CFU/g) were cultured from lung tissue samples compared to higher numbers (10^8–10^9 CFU/g) found in the lungs of placebo-treated animals (Table 6). Taken together, these findings indicate highly effective bacterial killing by plazomicin. Despite the large reduction in viable bacteria, however, lung damage was extensive and was considered the primary cause of death. Because the placebo-treated animals were succumbing at the same time as this animal, we are able to observe the impact of a single therapeutic dose of plazomicin on bacterial burden; compared to the median value of the placebo-treated

Table 2
Bacterial burden at time of death in placebo-treated animals

Animal	Lung	CFU/g						CFU/mL
		Spleen	Liver	Kidney	TBLN[a]	Heart	Brain	Blood
B6077	5.9×10^8	6.1×10^9	7.2×10^8	1.2×10^8	3.6×10^9	Nd[b]	Nd	Ns[c]
B6397	6.5×10^9	2.2×10^{10}	4.2×10^8	3.0×10^8	8.7×10^9	5.5×10^8	1.6×10^7	Ns
B6343	1.7×10^8	9.3×10^9	2.4×10^{10}	1.2×10^9	2.2×10^7	2.1×10^9	3.7×10^9	3.0×10^9
B6394	5.7×10^8	1.4×10^9	2.2×10^7	3.1×10^6	1.2×10^{10}	4.4×10^5	7.4×10^4	3.7×10^6
B6560	3.0×10^9	2.0×10^8	9.4×10^8	1.5×10^7	9.7×10^9	5.9×10^7	4.6×10^5	1.2×10^7
C2038	7.4×10^8	1.9×10^7	1.4×10^9	1.4×10^9	1.4×10^{10}	1.2×10^7	1.9×10^5	Ns
B6858	1.7×10^9	1.7×10^9	7.2×10^7	7.1×10^7	5.3×10^{10}	9.8×10^7	1.9×10^6	8.0×10^6
B6995	2.8×10^9	9.4×10^8	9.2×10^7	1.1×10^7	1.4×10^{10}	9.4×10^5	4.7×10^4	Ns
7514	7.9×10^8	1.9×10^9	9.0×10^8	1.7×10^8	4.6×10^9	2.6×10^7	5.3×10^7	Ns
8044	3.2×10^9	1.5×10^9	2.1×10^8	4.8×10^7	3.4×10^8	1.7×10^7	3.7×10^6	Ns
8058	2.7×10^9	1.4×10^{10}	6.1×10^8	7.0×10^7	6.7×10^9	8.5×10^7	1.2×10^7	5.2×10^8
7694	5.8×10^9	3.3×10^8	1.7×10^8	5.4×10^6	1.1×10^9	1.7×10^6	1.6×10^5	Ns

[a] Tracheobronchial lymph nodes.
[b] Nd – not determined. Samples were not obtained for this animal as the protocol was amended after gross observations were taken.
[c] Ns – no sample. Animal was found dead therefore terminal blood samples were not available.

Table 3
Clinical chemistry and hematology pre-study and at time of death

Animal	BUN[a] (mg/dL)		Creatinine (mg/dL)		GGT[b] (IU/L)		ALT[c] (IU/L)		LDH[d] (IU/L)		WBC[e] count ($10^3/\mu L$)	
	Pre	Terminal	Pre	Terminal	Pre	Terminal	Pre	Terminal	Pre	Terminal	Pre	Terminal
B6343	13	30	0.6	1.2	36	55	31	522	326	3890	4.1	33.5
B6394	19	46	0.5	1.4	96	220	40	686	539	6890	6.8	21.1
B6560	22	52	0.7	2.4	90	194	155	Nd[f]	465	Nd	9.1	47.4
B6858	19	41	0.8	2.2	45	112	27	90	209	2496	7.8	50.5
B6995	22	39	0.7	1.8	45	71	51	121	191	1824	6.0	23.4
8058	14	32	0.8	3.6	61	Nd	74	80	414	6510	5.7	45.4
7694	17	61	0.6	1.5	32	7	58	72	264	2525	8.4	10.8

[a] Blood urea nitrogen.
[b] Gamma glutamyl-transferase.
[c] Alanine aminotransferase.
[d] Lactate dehydrogenase.
[e] Total white blood cell count.
[f] Not determined – assay run failed quality control tests.

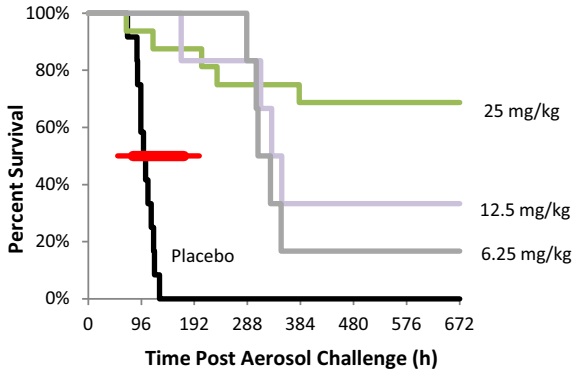

Figure 5a. Overall survival of plazomicin-treated animals, 5-day duration of therapy, compared to placebo. Red bar represents the time when a subset of (thinner portion) or all (thicker portion) surviving animals were receiving treatment.

animals, animal B6436 had 5–9 logs fewer bacteria in all tissues examined, indicating significant bacterial killing with a single dose.

Animal B6469 was found dead on day 5 post challenge, after 2 days of plazomicin treatment. Unlike animal B6426, this animal had only minimal to mild adverse findings in the lung. In contrast, moderate macrophage and neutrophil infiltration was observed in the meninges with associated hemorrhage, edema and blood vessel congestion. Based on these findings, meningitis was considered likely the cause of death in this animal. Animal B6873 was ruled moribund on day 7 post challenge, after 4 days of plazomicin treatment. This animal had multiple locally extensive areas of subacute inflammation in the left and right caudal lung lobes but other lobes showed only minimal interstitial mononuclear cell infiltration. These findings indicate that lung damage associated with aerosol

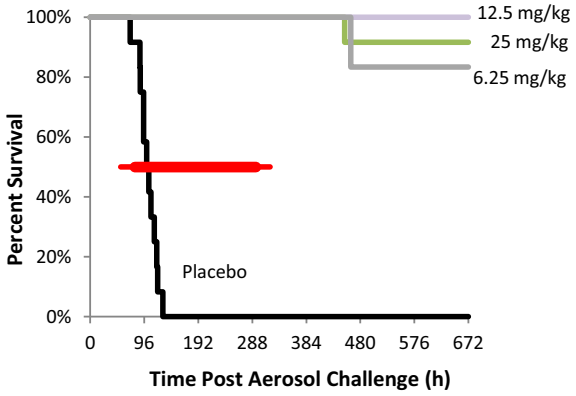

Figure 5b. Overall survival of plazomicin-treated animals, 10-day duration of therapy, compared to placebo. Red bar represents the time when a subset of (thinner portion) or all (thicker portion) surviving animals were receiving treatment.

Table 4
Survival by treatment group and study cohort

Treatment group	Study							Survival to study end p[a]	Survival time p[b]
	FY-105A		FY-105B		FY-036A				
	Cohort 1	Cohort 2	Cohort 1	Cohort 2	Cohort 1	Cohort 2	Totals		
Placebo	0/1	0/3	0/2	0/2	0/2	0/2	0/12	–	–
25 mg/kg, 5 d	4/6	2/4	2/3	3/3			11/16	0.0003	<0.0001
12.5 mg/kg, 5 d			2/3	0/3			2/6	0.0980	0.0001
6.25 mg/kg, 5 d			1/3	0/3			1/6	0.3333	0.0001
25 mg/kg, 10 d	2/3	3/3			3/3	3/3	11/12	<0.0001	<0.0001
12.5 mg/kg, 10 d					3/3	3/3	6/6	<0.0001	0.0001
6.25 mg/kg, 10 d					2/3	3/3	5/6	0.0007	0.0001

[a] Two-sided Fisher's exact test comparing treatment arm to placebo arm.
[b] Log-rank test comparing treatment arm to placebo arm, survival time through 28 days.

Table 5
Treatment duration effect on survival to study end[a]

Dose (mg/kg)	Survival with plazomicin treatment		p[b]
	5 day	10 day	
25	11/14	11/12	0.60
12.5	2/5	6/6	0.06
6.25	1/6	5/6	0.08

[a] Animals that received all scheduled plazomicin doses.
[b] Two-sided Fisher's exact test comparing 10-day treatment arm to 5-day treatment arm.

infection was limited by plazomicin administration. Similar to animal B6469, neutrophil infiltration and bacteria were observed in the meninges and choroid. Meningitis was considered the cause of the moribund clinical signs observed in this animal. Thus, animals B6469 and B6873 appeared to follow a similar progression. Both showed marked improvements in disease with respect to bacterial burden in all tissues (5–9 log reduction relative to median placebo control animals) as well as gross histopathological findings, however, both succumbed to plague meningitis.

3.6.2. Deaths post-therapy cessation

Thirteen animals died after completion of plazomicin therapy. All but one of these deaths were in animals that received either the lowest dose or short duration therapy. Ten of these 13 animals had an assigned cause of death of pneumonia and most of those animals were in the low- and mid-dose groups with short treatment duration (Table 6). In these animals, terminal necropsy suggested inadequate sterilization of the lungs that led to regrowth of bacteria and re-establishment of disease as the cause for failure.

We considered two possible reasons for treatment failure: failure to eradicate initial infection and emergence of resistance. The data more strongly favor failure to eradicate as the cause of treatment failure because at 10 days' duration, even the lowest dose was highly effective compared to shorter course therapy at this same dose. If emergence of resistance was the predominant cause for failure, we would have expected increased doses, but not necessarily longer durations of therapy, to play a major role in improving outcomes. To directly address the two hypotheses, we profiled three *Y. pestis* isolates from the lung tissue of animal 08047 which was given the low dose of plazomicin for 10 days and succumbed on day 19 post infection. All three isolates had identical MICs compared to the parent Colorado 92 strain (0.5 mg/L), supporting

Table 6
Bacterial burden at time of death in plazomicin-treated animals

Animal	Cause of death	Treatment arm	CFU/g							CFU/mL
			Lung	Spleen	Liver	Kidney	TBLN[a]	Heart	Brain	Blood
		Placebo (median)	7.7×10^8	1.6×10^9	5.2×10^8	7.1×10^7	7.7×10^9	2.6×10^7	1.9×10^6	1.2×10^7
B6436	Lung damage^{DOT}	25 mg/kg, 5 d	4.4×10^c	BLD[b]	BLD	BLD	3.0×10^b	Nd[c]	Nd	BLD
B6469	Meningitis^{DOT}	25 mg/kg, 5 d	2.1×10^b	4.6×10^a	BLD	BLD	BLD	Nd	Nd	NS[d]
B6873	Meningitis^{DOT}	12.5 mg/kg, 5 d	1.6×10^c	BLD	BLD	BLD	BLD	BLD	4.4×10^e	BLD
B6106	Meningitis	25 mg/kg, 5 d	1.9×10^d	3.1×10^c	9.5×10^c	1.7×10^c	BLD	BLD	6.4×10^7	4×10^a
B6872	CNS[e] infection	25 mg/kg, 5 d	BLD	BLD	BLD	BLD	BLD	BLD	4.1×10^6	BLD
B6127	Meningitis	25 mg/kg, 10 d	BLD	BLD	4.4×10^a	BLD	BLD	9.4×10^a	3.9×10^c	BLD
B6415	Pneumonia/ sepsis	25 mg/kg, 5 d	3.2×10^9	9.0×10^d	2.0×10^6	1.5×10^e	7.1×10^7	6.2×10^e	3.9×10^d	NS
C2083	Resurgence of pneumonia	12.5 mg/kg, 5 d	3.7×10^6	1.5×10^7	4.5×10^9	3.1×10^6	8.0×10^9	1.5×10^7	2.2×10^6	2.4×10^e
B6672	Resurgence of pneumonia	12.5 mg/kg, 5 d	9.2×10^9	2.5×10^d	7.6×10^e	2.5×10^d	8.3×10^6	2.9×10^d	2.3×10^c	NS
B6783	Resurgence of pneumonia	12.5 mg/kg, 5 d	2.8×10^9	1.0×10^e	8.4×10^6	7.6×10^c	6.3×10^9	8.0×10^c	3.3×10^b	2.7×10^b
B6861	Pneumonia	6.25 mg/kg, 5 d	1.2×10^8	9.5×10^d	1.6×10^e	1.1×10^d	1.2×10^8	1.6×10^e	1.2×10^e	NS
C2149	Pneumonia	6.25 mg/kg, 5 d	1.0×10^9	3.0×10^d	1.0×10^6	1.6×10^e	1.0×10^9	9.7×10^6	4.6×10^d	NS
B6889	Pneumonia	6.25 mg/kg, 5 d	2.2×10^9	6.2×10^6	2.2×10^7	1.1×10^6	3.6×10^9	1.0×10^6	8.0×10^d	9.3×10^e
B6960	Pneumonia	6.25 mg/kg, 5 d	3.0×10^9	2.3×10^d	3.8×10^d	7.2×10^c	1.6×10^7	1.2×10^6	BLD	NS
B6988	Pneumonia	6.25 mg/kg, 5 d	3.2×10^8	1.7×10^e	1.7×10^d	2.3×10^e	6.6×10^6	4.5×10^6	1.1×10^b	1.1×10^b
8047	Pneumonia	6.25 mg/kg, 10 d	6.6×10^9	1.9×10^e	1.3×10^6	3.2×10^d	2.0×10^9	2.0×10^7	6.0×10^d	NS

DOT – died while on therapy.
[a] Tracheobronchial lymph nodes.
[b] BLD – below limit of detection/no bacteria detected.
[c] Nd – not determined. Samples were not obtained for this animal as the protocol was amended after gross observations were taken.
[d] Ns – no sample. Animal was found dead therefore terminal blood samples were not available.
[e] CNS – central nervous system.

failure to eradicate due to insufficient dose/duration at the lowest dose as the cause for treatment failure.

The three non-pneumonia deaths that occurred post-therapy cessation were attributed to meningitis/central nervous system infection (Table 6). In all cases, bacteria were observed in the brain while marked reductions in bacterial burden were observed in other tissues. Animal B6106 was an exception in that it had moderate counts (~104 CFU/g) in the lung suggesting that if the animal did not succumb to the meningitis it may have suffered from a resurgent pneumonia.

One of the meningitis-attributed deaths was animal B6127, which was the lone death in the plazomicin high-dose/duration group. This animal had a notable history in the study. Between aerosol exposure and treatment, the animal's implanted temperature probe failed and therefore an alternative temperature measure was employed. This potentially delayed therapy initiation significantly, although it is difficult to know by how long. Histopathological examination of this animal revealed it had stomach ulcers and hematology findings that indicated stress contributed to the outcome. The animal had weight loss from day 7 post-challenge, worsening from approximately day 14 and developed inappetence and dehydration, attributed, in retrospect to worsening meningitis. Terminal serum chemistry showed elevated blood urea nitrogen. The higher blood urea nitrogen taken together with serum electrolyte abnormalities and moderate tubule damage in the kidney by histologic examination suggested that the animal had renal uremia with hyperchloremic metabolic acidosis and may also have had prerenal uremia because of dehydration. These factors contributed to and exacerbated the outcome. Serum chemistry physiologic and metabolic alterations in this animal may be explained by both the kidney tubule damage and pathophysiologic impairment associated with meningitis. Renal tubule alterations were attributed to plazomicin administration and indicated tubule toxicity (nephrosis). Therefore we hypothesize that the further delayed treatment allowed for meningitis to establish as with other animals mentioned above, however the downstream impact was a more advanced state of disease prior to initiating therapy. Fluid replacement therapy and dose adjustment for impaired renal function, which would be reasonable clinical interventions, were not designed as part of this study but may have improved the outcome.

3.6.3. Surviving animals

Thirty-six plazomicin-treated animals survived through study end. The majority (33/36) were either in the 10-day duration groups (high-/mid-/low-dose) or the 5-day duration high-dose group, supporting the importance of both dose and duration on a successful outcome. All 36 animals had no detectable bacteria in blood or any tissue tested. Serum chemistry and hematology parameters returned to baseline for all animals. Results for the 25 mg/kg 10-day treatment group are shown in Table 7.

Histopathological examination of the survivors, in general, revealed minimal to mild residual and resolving alterations in the lung. Overall, histologic findings in the lung, at the organ level, were considered non-adverse to minimally adverse. Evidence of slightly lower efficacy of the 6.25 mg/kg 10-day regimen was evident due to liver inflammatory cell infiltrates in animals at scheduled necropsy. There were no alterations in organ weights in any group. Residual and resolving kidney tubule atrophy alterations in a subset of animals in the 25 mg/kg 10-day and 12.5 mg/kg 10-day groups were focal, multiple and minimal. Alterations were considered non-adverse. They were considered possibly associated with plazomicin administration, but a dose–response was not observed. In fact, slightly more evident alterations in two animals in the low 6.25 mg/kg 10-day group suggest that a more persistent *Y. pestis* bacteremia and systemic infection contributed to the kidney findings.

4. Discussion

Y. pestis is considered to be one of the most lethal possible bioweapons. There are only two FDA-approved agents for the treatment of pneumonic plague and both share common resistance mechanisms.[6,7] This study demonstrated the efficacy of a new antibiotic, plazomicin, in the treatment of lethal pneumonic plague in a rigorous nonhuman primate model. All animals who received placebo died, while 36 of 52 animals that received plazomicin survived until study end on day 28 post-exposure. Demonstration of efficacy depended on both the dose of plazomicin and the duration of treatment. In this work we defined the minimum dose and duration of plazomicin therapy required to successfully treat advanced pneumonic plague in the most widely accepted model, the African Green monkey. To ensure the model was sufficiently challenging, animals were treated only when the pneumonia had progressed to a systemic disease and bacteremia was confirmed in 60 of 64 animals tested using fever as a surrogate marker for systemic disease progression. In future pivotal studies, randomization to treatment arms could be done post-fever detection and it would be wise to slightly over-power each arm to accommodate the occasional animal in which bacteremia is below detection limits.

This work provided a unique opportunity to study the efficacy of plazomicin in a pre-clinical model that is more akin to human infection than the models classically used to characterize antibiotic efficacy. Classic pre-clinical efficacy studies rely on immunocompromised rodent models that mostly examine dose/effect relationships in the absence of an appreciation for the host-pathogen interaction.[28] These models serve the field well in setting doses for acute response (initial bacterial kill) but fall short in three critical, and interrelated, areas: (1) they do not test how the drug performs in the presence of very high bacterial burdens where a large resistant sub-population is likely to be present for many drugs; (2) they do not test the impact of duration of therapy and (3) they fail to examine physiologically critical organ and tissue types associated with the clinical manifestation of infection. For example, a classic neutropenic mouse thigh model might examine 10^6–10^7 total bacteria in an isolated site (thigh tissue), whereas in this study bacterial burdens in excess of 10^{11} total organisms are achieved in a multi-organ infection setting. There are few opportunities in in vivo systems to challenge a drug under these conditions short of actual human clinical trials. Furthermore, in human trials of severe infections, the need for combination therapy to match current standards of care complicates interpretation. Therefore the ability to study a drug as a monotherapy under challenging infection conditions may be unique to these types of models.

Based on overall survival, the minimum therapeutic daily dose of plazomicin was 6.25 mg/kg when given for sufficient duration. This dose is equivalent to ~3 mg/kg in humans, 5-fold below the current clinically proposed dose. We demonstrated the importance of therapy duration, showing a trend toward superiority with 10 compared to 5 days of therapy at the mid- and low-dose levels. High-dose therapy (25 mg/kg, ~12 mg/kg equivalent dose in humans), even at short (5-day) duration, was able to provide significant protection, which supports claims in the literature that if therapy intensity is sufficiently high, shorter courses of treatment can be successful.[29] This may be particularly relevant for an extremely virulent agent like *Y. pestis* where a small bacterial exposure leads to disease and thus even a small remaining reservoir of viable bacteria post-treatment could lead to disease relapse. With an agent such as plazomicin that is rapidly bactericidal,[30] protection can be achieved provided the dose is sufficiently high as

Table 7
Serum chemistry and hematology results for survivors treated with 25 mg/kg plazomicin for 10 days

Animal	BUN[a] (mg/dL)		Creatinine (mg/dL)		GGT[b] (IU/L)		ALT[c] (IU/L)		LDH[d] (IU/L)		WBC[e] Count (10^3/μL)	
	Pre	Terminal	Pre	Terminal	Pre	Terminal	Pre	Terminal	Pre	Terminal	Pre	Terminal
B5994	15	18	0.6	0.5	35	38	62	46	364	403	8.0	4.5
B6408	22	20	0.5	0.4	53	56	109	94	249	304	6.9	5.5
B6291	24	21	0.6	0.5	77	102	47	68	212	259	12.0	5.9
B6140	24	27	0.8	0.7	55	39	180	71	290	379	6.8	7.2
B6428	16	26	0.8	0.6	31	27	83	60	238	299	6.2	6.0
7972	13	24	0.8	0.7	46	38	30	36	381	398	6.9	7.6
7929	15	17	1.0	0.9	44	16	63	41	653	496	10.9	8.5
8067	13	21	0.8	0.8	40	26	45	50	287	478	10.0	9.4
7643	16	19	0.5	0.4	44	35	53	43	278	606	6.1	5.7
7337	15	18	0.7	0.5	67	63	55	44	310	469	7.5	5.0
7653	22	26	0.6	0.8	37	27	50	48	319	426	7.5	5.8

[a] Blood urea nitrogen.
[b] Gamma glutamyl-transferase.
[c] Alanine aminotransferase.
[d] Lactate dehydrogenase.
[e] Total white blood cell count.

demonstrated in the high-dose group receiving 5 days of therapy. These findings suggest that relatively short course therapy with the current clinical dose of plazomicin may be an effective treatment for human plague.

In our studies, efforts were made to minimize the number of animals receiving placebo. Given the possible variance in animal health history, the complexity of generating the bioaerosols, and the paucity of available data regarding this animal model (our studies were designed and initiated in 2010 prior to the public release of the levofloxacin and moxifloxacin studies), we felt the inclusion of two males and two females per study was imperative. With the large body of data now available from our studies and those of the fluoroquinolones regarding the natural history of placebo-treated pneumonic plague in AGMs, we suggest that future studies in this model consider relying on this historical dataset as a control rather than utilize additional placebo-treated animals.

Given the absence of robust human clinical efficacy data in this indication, study of an adequate dose response in animal models is essential. This mandates dosing to levels that demonstrate compromised/reduced efficacy and/or decreased survival rates, so that better PK/PD relationships can be established. For the two currently approved drugs, levofloxacin and moxifloxacin, no dose response information has been communicated, therefore it is difficult to predict and at what exposure (or *Y. pestis* MIC) efficacy would be compromised. This is a critical knowledge gap because the PK of these drugs[25,31] is altered dramatically in critically ill patients.[24–26,32] Our current analysis supports that the plazomicin exposures in *Y. pestis*-infected AGMs are similar to those observed in healthy AGMs. Plazomicin PK data from critically-ill patients infected with CRE will be included in the plazomicin population PK model and this information may be used to inform recommended dose/exposure for future pneumonic plague pivotal studies.

Both levofloxacin and moxifloxacin provided impressive benefit in the AGM model, with 92% and 100% survival at the one dose level tested, administered for a 10-day duration.[6,11,12] Plazomicin achieved similar efficacy, resulting in 92%, 100% and 83% survival of animals at the high, mid- and low doses administered for 10 days. During the course of our studies, we identified meningitis as a significant contributor to treatment failure. The observation of plague meningitis in this model was unexpected. Plague meningitis is rare in humans (approximately 6% of cases)[33] and has not yet been reported by others in the AGM infection model, including in the fluoroquinolone studies. Fluoroquinolones have higher penetration into cerebral spinal fluid than aminoglycosides[34–36] and therefore may have superior efficacy against plague meningitis. Our data and the published levofloxacin and moxifloxacin studies support this. It is important to highlight that although the fluoroquinolones are able to reach therapeutic levels in the cerebral spinal fluid, they are at much lower concentrations than in the bloodstream.[36] Therefore it is even more critical to understand the exposures at which efficacy of these drugs begins to wane, to inform when to deploy other assistive countermeasures such as chloramphenicol, which is the recommended treatment when plague meningitis is suspected.[33]

Findings of plague meningitis motivated us to modify the study protocols going forward to sample brain tissue in greater detail. From this we determined that *Y. pestis* are readily detected in the brains of most animals at time of death. For placebo-treated animals we hypothesize that due to the rapid progression of pneumonia and sepsis, meningitis is never able to fully develop as an attributable cause of death. We further hypothesize that in animals where therapy is initiated too late and/or where inadequate systemic bacterial eradication occurs, *Y. pestis* is able to establish an infection in the central nervous system where the majority of antibiotics, including aminoglycosides like plazomicin, have limited exposure. This in turn, leaves bacterial proliferation unchecked locally, leading to development of meningitis and potentially serving as a reservoir for bacterial spread once therapy is stopped.

In all other tissues, including the lung, plazomicin was highly effective. The efficacy of aminoglycosides in pneumonia has been questioned in the literature; hypotheses such as poor lung penetration, chelation by lysed white blood cell DNA, and the acidic environment in the lung have been proposed as reasons for reduced efficacy.[29] However, little clinical or pre-clinical primary data are available to support these claims. Our studies of plazomicin, and the legacy aminoglycosides, support that they have excellent activity in multiple preclinical lung infection models.[27,37–40] In fact, prior to the approval of levofloxacin and moxifloxacin, gentamicin and streptomycin were the primary recommended therapies for the treatment of plague.[3]

A potential explanation for the discord could be that early aminoglycoside usage employed very low, sub-optimal doses and dosing schedules. For example in the 1970s and 1980s, gentamicin was dosed ~1 mg/kg every 8 h compared to modern optimized dosing of 5–7 mg/kg once daily.[29,41] Two published comparative studies of aztreonam (a monobactam antibiotic) and the aminoglycoside tobramycin concluded that tobramycin is inferior to aztreonam for the treatment of Gram-negative pneumonia.[42,43] A third study published in the same timeframe demonstrates equivalence

of these two drugs.[44] In all 3 studies, the aztreonam dose was between 3000 and 8000 mg per day while the tobramycin dose was 80 mg every 8 h. Given the current understanding that once daily dosing of aminoglycosides is superior both in terms of safety and efficacy,[29,41,45] these prior clinical observations may be misleading.

In addition to under-dosing, aminoglycoside susceptibility breakpoints were (and in some cases still are) too high based on our modern understanding of the PK/PD relationship of the class. Based on a thorough evaluation of PK–PD target attainment analyses, and available clinical outcomes data, USCAST has recently proposed lowering the susceptibility breakpoints for all currently marketed aminoglycosides, (USCast002 v1.1, www.uscast.org), supporting the notion that historical breakpoints are too high. The combination of these factors enabled legacy aminoglycoside use with sub-optimal doses, and for organisms whose MICs were so high that unacceptably low rates of target attainment would be expected, especially in the case of the lung pathogen *Pseudomonas aeruginosa*.

In conclusion, plazomicin represents a valuable addition to the armamentarium against pneumonic plague. The fluoroquinolones are a highly attractive countermeasure given the availability of both IV and oral formulations and their superior cerebral spinal fluid penetration. However, additional antibiotic classes with diverse mechanisms of action are required to prevent over-reliance on a single class to which resistance may be easily bred. Based on the data generated here, the addition of plazomicin to the current countermeasure options is warranted and these studies will inform an optimal design for pivotal trials in this indication.

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